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A CYTOLOGICAL ANALYSIS OF FERTILIZATION IN *CHAETOPTERUS PERGAMENTACEUS*[3]

WINSTON A. ANDERSON[1] AND WILLIAM R. ECKBERG[1,2]

[1]*Department of Zoology, Howard University, Washington, DC 20059, and* [2]*Marine Biological Laboratory, Woods Hole, MA 02543*

ABSTRACT

We have examined sperm-egg interaction in *Chaetopterus pergamentaceus* by electron microscopy. The initial contact between sperm and egg involved the membrane of the unreacted acrosome and either the tips of egg microvilli which penetrated the vitelline layer or jelly emanating from the tips of the microvilli. This resulted in an acrosome reaction and fusion between the inner acrosomal membrane and the tip of the microvillus. Sperm did not produce acrosomal processes like those of many other invertebrates, and no part of the sperm penetrated the vitelline layer until the sperm was incorporated into the fertilization cone. The fertilization cone was very small and was composed of egg microvilli. The sperm nucleus and mitochondrion were incorporated into the fertilization cone, but a recognizable sperm mitochondrion could not subsequently be seen in the egg cytoplasm. Although the axoneme of the sperm tail was present in the fertilization cone at early stages of sperm penetration, the sperm tail evidently detached in the later stages of incorporation because it could not be seen in the zygote cytoplasm after sperm incorporation. The sperm chromatin decondensed uniformly and became surrounded by a typical nuclear envelope. The results indicate that *Chaetopterus* provides an example of a previously undescribed model for sperm penetration of egg vestments in which the sperm needs neither to produce an acrosomal process nor to liberate vitelline layer lysins because it penetrates the vitelline layer passively after incorporation into the egg cytoplasm.

INTRODUCTION

Fertilization is characterized by a sequence of events. A gamete interaction triggers the acrosomal reaction that initiates initial sperm-egg attachment, subsequent gamete membrane fusion, zygote formation, and egg activation. Sperm incorporation ensues, and finally the genetic material of the two gametes combines.

In Spiralians, studies of gamete interactions have been limited to the molluscs *Barnea* (Pasteels, 1965), *Mytilus* (Longo and Anderson, 1969), *Spisula* (Longo and Anderson, 1970) and *Haliotis* (Lewis *et al.*, 1982), the annelids *Hydroides* (Colwin and Colwin, 1961a, b) and *Nereis* (Fallon and Austin, 1967) and the echiurid, *Urechis* (Tyler, 1965; Paul and Gould-Somero, 1976). Sperm-egg interaction in these forms appears to follow several plans.

In *Hydroides* and *Haliotis*, the sperm undergoes an acrosome reaction in association with the outer surface of the vitelline layer and penetrates the vitelline layer with the assistance of sperm lysins which partially dissolve the vitelline layer. In *Hydroides* (Colwin and Colwin, 1960), the mechanism of this penetration is not

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known, but in *Haliotis,* the sperm lysin evidently acts by a non-enzymatic mechanism (Lewis *et al.,* 1982). In *Barnea* (Pasteels, 1965), *Urechis* (Tyler, 1965; Paul and Gould-Somero, 1976) and probably *Spisula* (Longo. 1976) a sperm acrosomal filament fuses with an egg microvillus and the sperm nucleus penetrates the vitelline layer after being incorporated into the fertilization cone.

We obtained evidence that the *Chaetopterus* vitelline layer played a role in preventing polyspermy, but did so without being structurally or functionally changed after fertilization (Eckberg and Anderson, 1983). Additionally, in preliminary studies, we did not obtain evidence for sperm lytic activity against the vitelline layer. Therefore, we initiated a study of sperm-egg interaction in this species. The results showed that the fertilizing sperm fuses with the tip of one or more egg microvilli which extend beyond the vitelline layer and is surrounded by a fertilization cone. We also found that egg microvilli retract from the vitelline layer after fertilization. Therefore the vitelline layer of the fertilized egg can become a physical barrier to sperm-egg fusion without being structurally or functionally altered by fertilization.

Materials and Methods

Gametes were obtained and handled, fixed for 1 h at room temperature in 5% glutaraldehyde, 4% paraformaldehyde, 0.1 *M* sodium cacodylate, pH 7.8 in artificial sea water, and processed for light and electron microscopy as described (Eckberg, 1981a).

Inseminated eggs were fixed at intervals after fertilization (0.5, 1, 2, 3, 5, 9, and 14 min). Male pronuclear formation was complete by 14 min. Although the eggs examined in this study were polyspermic due to heavy insemination, sperm associated with the vitelline layer more than 1 min after insemination were supernumerary because this species has a complete block to sperm penetration by this time (Eckberg and Anderson, 1983). Polyspermic eggs develop synchronously with controls up to the time of cleavage. Although they fail to divide, they undergo differentiation without cleavage (Lillie, 1902; Eckberg, 1981b; Eckberg and Kang, 1981). Therefore the events of fertilization in such polyspermic eggs are very likely to be the same as those in monospermic eggs.

Results

Oocyte surface

The *Chaetopterus* egg is normally inseminated at the first meiotic metaphase. The cytoplasmic organization of the oocyte at this stage has been described (Eckberg, 1981a). Since the sperm interacts with the vitelline layer and oocyte surface, this region will be described more fully here. The vitelline layer is fibrous and is organized into three distinct regions: an inner region composed of a dense fibrous meshwork, a middle region composed of fibers oriented parallel to the oocyte surface, and an outer region of electron-dense granules interspersed with the tips of microvilli (Figs. 1, 2). This is covered by an outer diffuse "jelly" layer (Figs. 1, 2). Jelly filaments originate from the granules and the microvillar tips.

Sperm

Mature sperm consist of a head and midpiece about 1 μm $\times$ 4 μm and a long flagellum (Fig. 3). Transverse sections (not shown) reveal a single mitochondrion surrounding a centriole pair which serves as the origin of the flagellum. The acro-

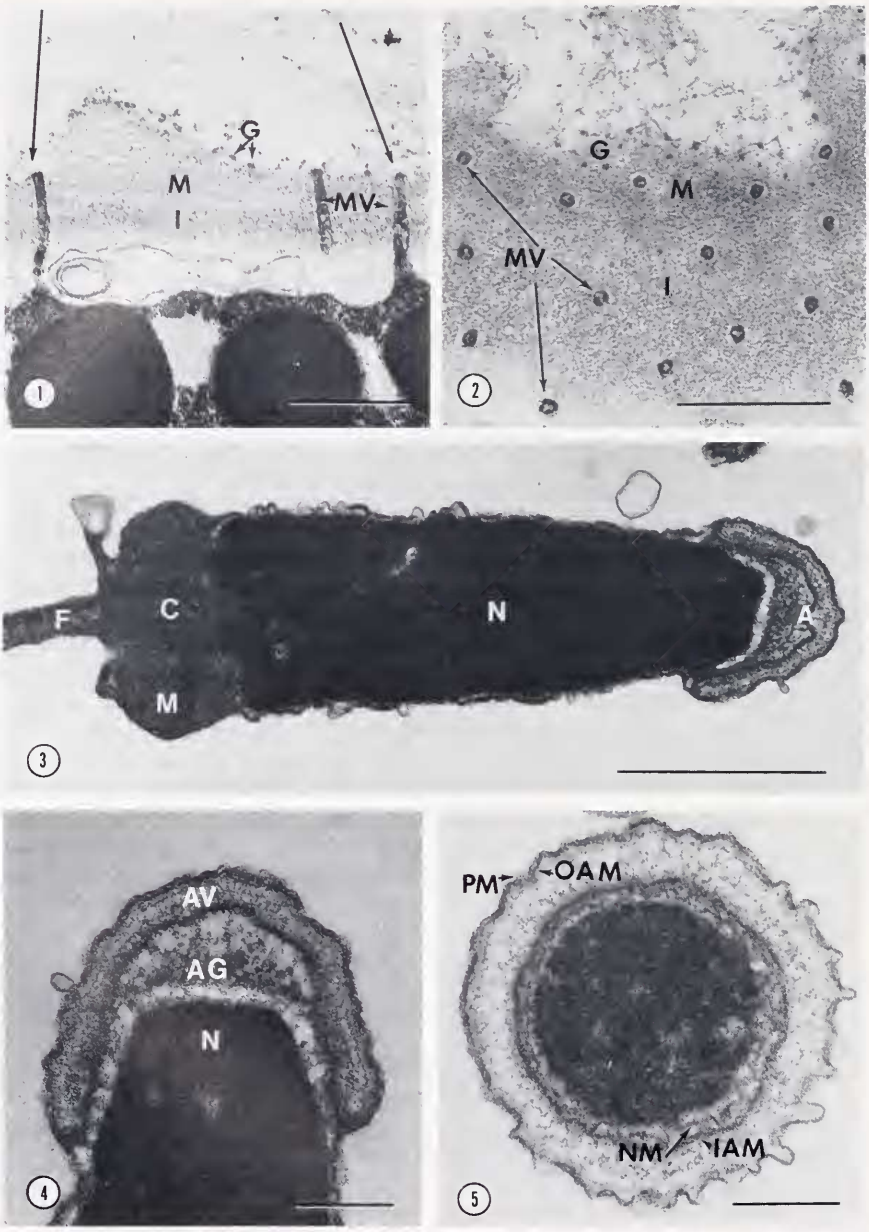

FIGURE 1. Surface of an unfertilized egg of *Chaetopterus*. Note the three regions of the vitelline layer: I = inner dense layer, M = middle layer, G = granules comprising the outer layer. Note also that microvilli (MV) penetrate the vitelline layer completely. Also note the fibrillar jelly coat originating from the tips of the microvilli and the granules of the vitelline layer (arrows). Bar = 1 μm.

FIGURE 2. Tangential section of the vitelline layer and jelly coat of an unfertilized egg. Symbols are as given in the legend to Figure 1. Note that the granules of the outer region of the vitelline layer are numerous between the tips of the microvilli. Bar = 1 μm.

FIGURE 3. Longitudinal section of a *Chaetopterus* sperm. A = acrosome, N = nucleus, M = mitochondrion, C = centriole, F = flagellum. Bar = 1 μm.

FIGURE 4. Longitudinal section through the acrosomal region of a *Chaetopterus* sperm. Note the

somal region consists of a cup-like acrosomal vesicle containing fibrous material associated with its membranes and a region of granular material between the acrosomal vesicle and the apex of the nucleus. The acrosomal vesicle also covers the apical end of the sperm nucleus (Figs. 4, 5).

Gamete contact and fusion

The initial contact between sperm and egg involves the outer acrosomal membrane and the jelly in association with the microvilli (Fig. 6). Sperm with reacted acrosomes are oriented perpendicular to the oocyte surface (erect) (Fig. 7). The acrosome reaction involves the opening of the acrosomal vesicle and results in fusion between the inner acrosomal membrane and an egg microvillus (Fig. 8). Sperm do not produce acrosomal processes, and the tiny membranous projections which formed as the result of the acrosome reaction did not penetrate the vitelline layer.

Sperm incorporation

This process involves the formation of a tiny fertilization cone, barely visible in light micrographs (Fig. 8 inset), which consists of a few thickened microvilli surrounding the sperm (Figs. 9, 10). These microvilli contain longitudinal microfilament bundles (Fig. 10). The nuclear membrane of the newly-incorporated sperm becomes vesiculated and the sperm chromatin begins to disperse (Fig. 11).

After incorporation of the sperm head, a sperm tail protrudes from some, but not all residual fertilization cones (Fig. 10 insets). However, we never observed sperm flagella within the zygote other than short segments in the fertilization cone (Fig. 9). Nor did we observe recognizable sperm mitochondria in the zygote cytoplasm subsequent to sperm incorporation.

Fertilized egg surface

The vitelline layer is structurally unchanged after fertilization. All three regions are present and structurally similar to those of the unfertilized egg. However, the egg microvilli are generally absent from the vitelline layer (Fig. 12). Where they are present, they are greatly reduced in number and do not penetrate to the surface of the vitelline layer.

Formation of the male pronucleus

Sperm chromatin decondenses completely and uniformly (Fig. 13), and the nuclear envelope disappears (Fig. 14). Decondensed chromatin is frequently associated with small granules similar, but not identical to, the lipid granules of the oocyte (Fig. 14, 15). After complete decondensation, membrane vesicles surround the chromatin (Fig. 15) and eventually coalesce into a typical annulate pronuclear envelope (Fig. 16).

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cuplike acrosomal vesicle (AV) containing fibrous material associated with the membranes and the granular material (AG) between the acrosomal vesicle and the nucleus. N = nucleus. Bar = 0.25 μm.

FIGURE 5. Transverse section through the acrosomal region of a *Chaetopterus* sperm. The plasma membrane (PM) is clearly separated from the outer acrosomal membrane (OAM) at a few points. The inner acrosomal membrane (IAM) is clearly separated from the nuclear membrane (NM). Fibrous material in the acrosomal vesicle can be seen, but the acrosomal granule is out of the plane of this section. Bar = 0.25 μm.

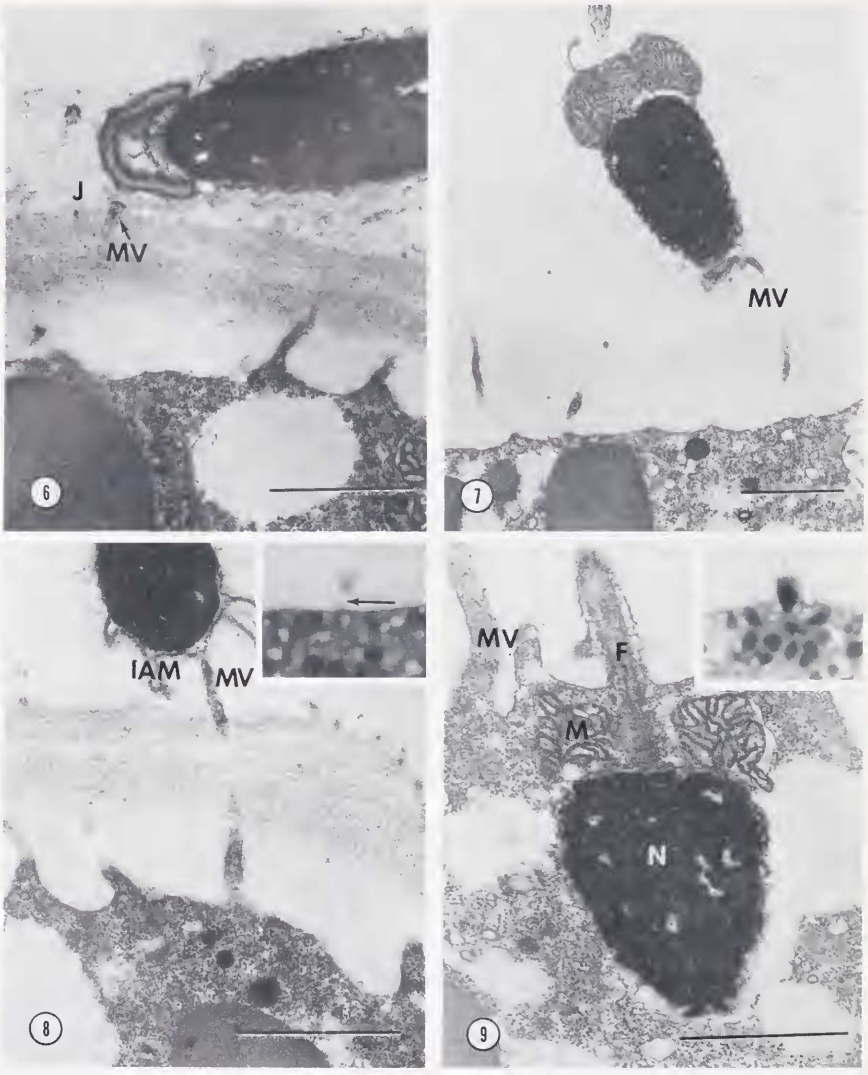

FIGURE 6. Initial interaction between a sperm and a microvillus prior to gamete fusion. Note that the plasma membrane over the acrosome is associated with a microvillus tip (MV) via the jelly (J). Bar = 1 μm.

FIGURE 7. Erection of a sperm following initiation of the acrosome reaction and attachment of the sperm to a microvillus (MV). Bar = 1 μm.

FIGURE 8. Gamete fusion involving the sperm inner acrosomal membrane (IAM) and an egg microvillus (MV). Bar = 1 μm. Inset: light micrograph showing a sperm attached to an egg microvillus which has thickened to the point where it is visible at this level of resolution and can thus be called a fertilization cone (arrow).

FIGURE 9. Sperm incorporation into the *Chaetopterus* egg. Note that the sperm nucleus (N), mitochondrion (M) and base of the flagellum (F) have all been incorporated. Note also the microvilli (MV) which surround the sperm and make up the small fertilization cone. Bar = 1 μm. Inset: light micrograph of a slightly earlier stage in fertilization cone formation showing several microvilli surrounding the incorporated sperm.

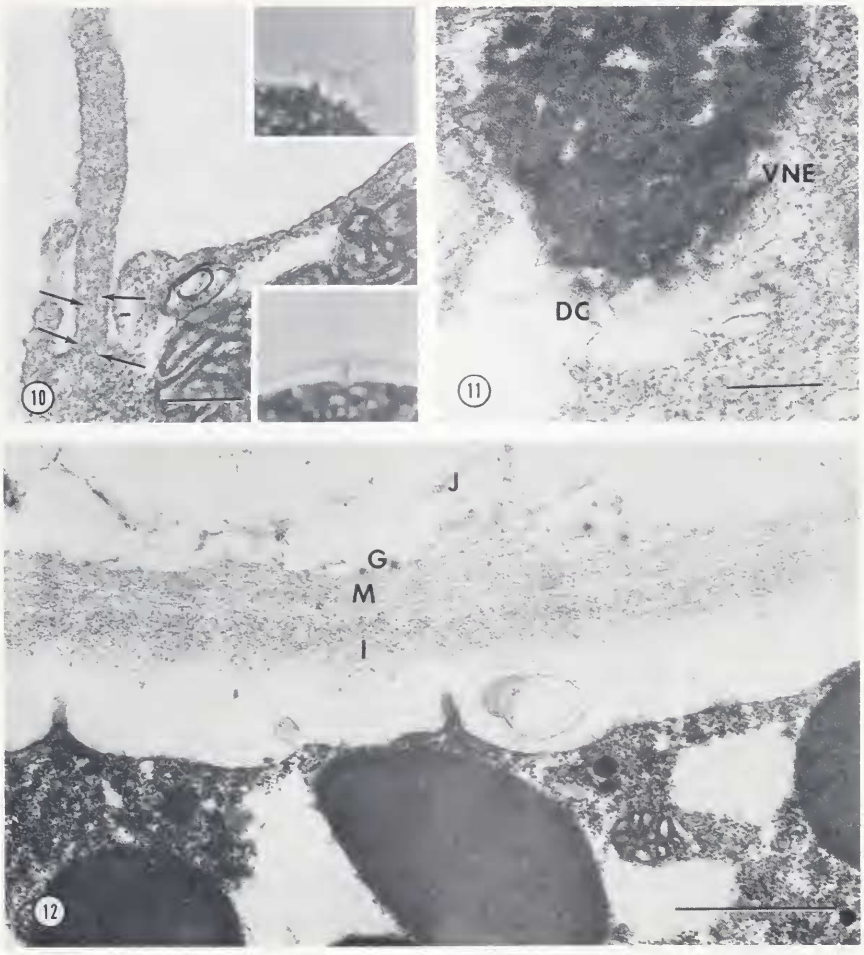

FIGURE 10. Higher magnification electron micrograph showing microfilaments (arrows) longitudinally-arranged in the microvilli of the fertilization cone. Bar = 0.25 μm. Insets: light micrographs showing late stages in fertilization cone formation. In the upper inset, the sperm tail still protrudes from the fertilization cone; in the lower inset, the sperm tail has been lost.

FIGURE 11. Nucleus of a newly-incorporated sperm. The nuclear envelope has become vesiculated (VNE) and the chromatin appears to be beginning to decondense (DC). Bar = 0.25 μm.

FIGURE 12. Surface of a fertilized egg 9 min after insemination. Note that the egg microvilli have shortened and no longer penetrate the vitelline layer, although all regions of the vitelline layer (I = inner, M = middle, G = granular) and the jelly (J) remain. Bar = 1 μm.

DISCUSSION

The *Chaetopterus* oocyte surface was similar to that observed in other species. The outer diffuse jelly coat was evidently the substance initially contacted by the sperm and appeared to originate from the granules at the ends of the microvilli and at the outer region of the vitelline layer. Similar granules appear at the initial contact points in *Urechis* (Tyler, 1965), *Nereis* (Fallon and Austin, 1967), and *Hydroides* (Colwin and Colwin, 1961a). These may originate during oogenesis as buds from the tips of oocyte microvilli (L. E. Franklin, data presented in Metz, 1967). This

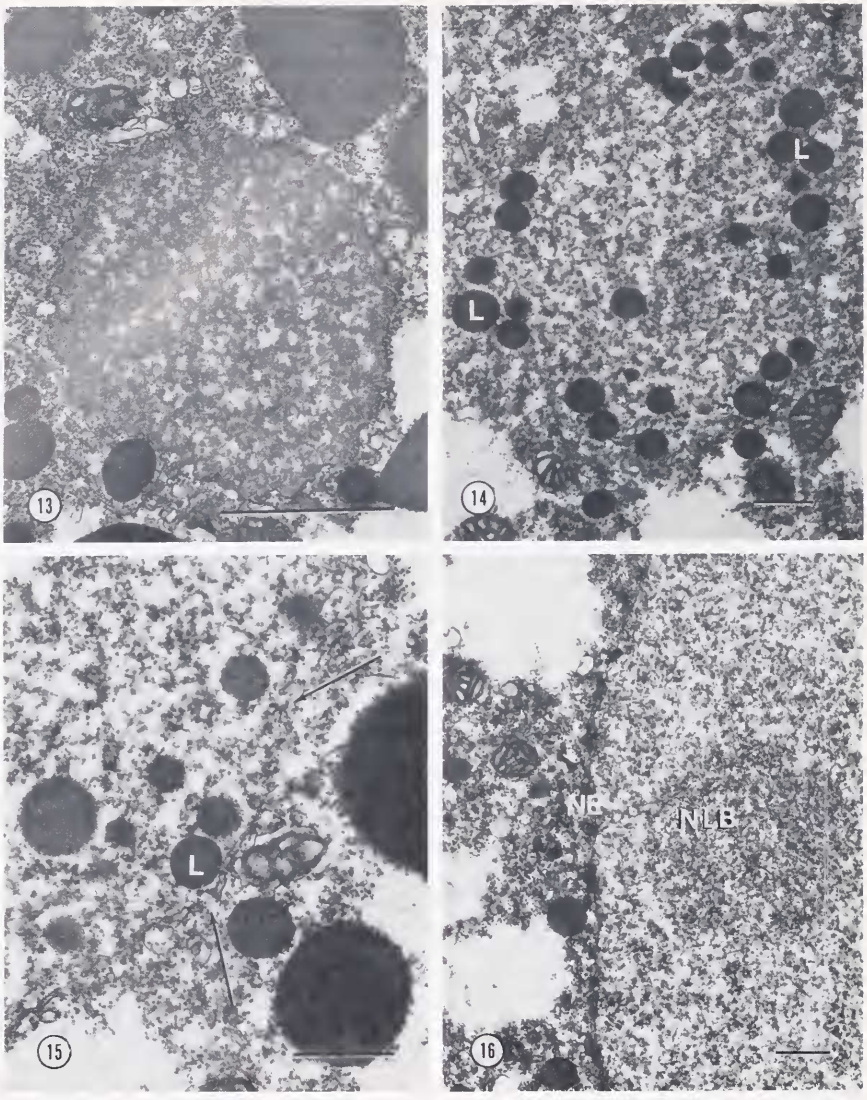

FIGURE 13. Decondensing sperm nucleus showing a stage slightly later than that in Figure 11. The sperm nuclear membrane remains vesiculated and the chromatin is decondensing uniformly throughout the nucleus. Bar = 1 μm.

FIGURE 14. Fully decondensed sperm nucleus, without a nuclear envelope, in association with lipid granules (L). Bar = 0.5 μm.

FIGURE 15. Fully-decondensed sperm nucleus showing association with lipid granules (L) and a vesiculated nuclear envelope (arrows). Bar = 0.5 μm.

FIGURE 16. Male pronucleus showing annulate nuclear envelope (NE) and a dense nucleolus-like body (NLB) in the pronucleus. Bar = 0.5 μm.

homology suggests similar function. We propose that these structures contain a receptor which initiates the acrosome reaction and is therefore analogous to the fucose-sulfate polysaccharide of sea urchin egg jelly (SeGall and Lennarz, 1979). Additional granules may initiate the acrosome reaction in supernumerary sperm (Eckberg and Anderson, 1983).

Gamete fusion took place via the inner acrosomal membrane of the sperm and the tips of egg microvilli. Sperm did not produce acrosomal processes. Other species which do not produce acrosomal processes generally fuse with the egg with the assistance of lysins which facilitate penetration of the vitelline layer (Colwin and Colwin, 1960; Lewis *et al.,* 1982). Such is evidently not the case in *Chaetopterus,* because (1) in preliminary experiments we could detect no evidence for sperm lysins, (2) sperm fused with the tips of egg microvilli which protruded through the vitelline layer, (3) in fertilized eggs such microvilli no longer penetrated the vitelline layer, (4) chemical disruption of the vitelline layer permitted refertilization (Eckberg and Anderson, 1983), presumably by making the oocyte surface available again to sperm, and (5) sperm were never observed to penetrate the vitelline layer until they were incorporated into the fertilization cone. The preceding observations also indicate that this microvillar retraction from the vitelline layer can provide a mechanism for a permanent block to polyspermy in this species.

Sperm of other species which produce acrosomal processes may (Pasteels, 1965; Tyler, 1965; Longo, 1976) or may not (Longo and Anderson, 1968) fuse with egg microvilli, but if they fuse with microvilli preferentially, they apparently do not fuse with the tips (Pasteels, 1965; Tyler, 1965). *Chaetopterus* thus provides an example of a previously undescribed model for sperm penetration of egg vestments in which the sperm needs neither to produce an acrosomal process nor to liberate vitelline layer lysins because it penetrates the vitelline layer passively after incorporation into the egg cytoplasm.

Sperm incorporation was mediated by a tiny fertilization cone (Morgan and Tyler, 1930), shown here to be composed of slightly thickened microvilli. Since such microvilli contained bundles of microfilaments, fertilization cone formation and action would appear similar in mechanism to that observed in other species (Tyler, 1965; Longo, 1978).

A recognizable sperm mitochondrion could not be seen in the zygote cytoplasm subsequent to incorporation. However, since it was present in the fertilization cone, the sperm mitochondrion must have been incorporated into the zygote. In *Mytilus,* the sperm mitochondrion reportedly becomes indistinguishable from egg mitochondria (Longo and Anderson, 1969). A similar situation may exist in *Chaetopterus.* This differs, however, from sea urchins, in which the sperm mitochondrion persists as an identifiable structure during cleavage and is metabolically active (Anderson, 1968; Anderson and Perotti, 1975).

The lack of complete incorporation of the sperm tail is similar to the situation in other spiralians (Tyler, 1965; Longo and Anderson, 1969, 1970), but different from that in sea urchins (Longo and Anderson, 1968) and mammals (Piko, 1969) in which sperm tails can be seen in the zygote cytoplasm long after fertilization. However, the sperm centriole is incorporated and sets up the first cleavage spindle (Mead, 1895).

Acknowledgments

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